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(54) Title: COATED MAGNETIC PARTICLES FOR USE IN SEPARATIONS

(57) Abstract

Magnetically responsive particles and their use in systems in which the separation of certain molecules from the surrounding medium is necessary or desirable are disclosed. The magnetically responsive particles consist of a metal, metal oxide or metal alloy core, coated with a polymer having attached thereto an organic functionality to which a variety of organic and/or biological molecules can be coupled. The particles can be dispersed in aqueous media without rapid gravitational settling and conveniently reclaimed from the media using a magnetic field. The magnetically responsive particles of the invention may be coupled to biological or organic molecules with affinity for, or the ability to adsorb, or which interact with certain other biological or organic molecules. Particles so coupled may be used in a variety of *in vitro* or *in vivo* systems involving separation steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses.

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COATED MAGNETIC PARTICLES FOR USE IN SEPARATIONS

Description

Background of the Invention

Magnetic separations in biological systems have been used as an alternative to gravitational or centrifugal separations. B.L., Hirschbein, et al., Chemtech, pp. 172-179 (1982); M. Pourfarzaneh, The Ligand Quarterly, 5(1):41-47 (1982); and P.J., and P., Dunhill, Enzyme Microb. Technol. 2:2-10 (1980). There are several advantages to using magnetically separable particles as supports for biological molecules such as enzymes, antibodies and other bioaffinity adsorbents. For example, when magnetic particles are used as solid phase supports in immobilized enzyme systems, the enzyme can be selectively recovered from the media, including media containing suspended solids, allowing recycling in enzyme reactors. P.J., Robinson, et al., Biotech. Bioeng., 15:603-606 (1973). When used as solid supports in immunoassays or other competitive binding assays, magnetic particles permit the reaction to occur under homogeneous conditions, which promotes optimal binding kinetics, minimally alters analyte-adsorbent equilibrium and facilitates separation of bound from unbound analyte, particularly as compared to centrifugation.

Centrifugal separations are time-consuming, require expensive and energy-consuming equipment and pose radiological, biological and physical hazards. Magnetic separations are relatively rapid and easy, requiring

simple equipment. The use of non-porous adsorbent-coupled magnetic particles in affinity chromatography systems allows better mass transfer and results in less fouling of the sample than in conventional affinity chromatography systems.

The practical development of magnetic separations has been hindered by several critical properties of the magnetic particles developed thus far. For example, large magnetic particles (e.g., having a mean diameter in solution greater than 10 microns) respond to weak magnetic fields and magnetic field gradients; however, they tend to settle rapidly, limiting their usefulness for reactions requiring homogeneous conditions. Large particles also have a more limited surface area per weight than smaller particles, so that less material can be coupled to them. Examples of large particles are those described by Robinson et al., in Biotech. Bioeng., 15:603-606 (1973), which are 50-125 microns in diameter; particles described by Mosbach and Anderson in Nature, 270:259-261 (1977), which are 60-140 microns in diameter and those described by Guesdon et al., in J. Allergy Clin. Immunol., 61(1):23-27 (1978) which are 50-160 microns in diameter.

Smaller magnetic particles have been described, for example, composite particles described by Hersh and Yaverbaum, in U.S. Patent No. 3,933,997, are ferromagnetic iron oxide (Fe_3O_4) carrier particles, which were reported to have diameters between 1.5 and 10 microns. However, based on the reported settling rate of 5 minutes and coupling capacity of only 12 milligrams of protein per gram of particles, the actual size of the particles

in solution is expected to be substantially greater than 10 microns. L.S. Hersh and S. Yaverbaum, Clin. Chem. Acts, 63:69-72 (1975).

Small magnetic particles (e.g., having a mean diameter in solution less than about 0.03 microns) can be kept in solution by thermal agitation and do not tend to settle spontaneously. However, the magnetic field and magnetic field gradient required to remove such particles from solution are large and require heavy and bulky magnets for generating these fields, which are inconvenient to use in bench top work. Magnets capable of generating magnetic fields in excess of 5000 Oersteds, for example, are typically required to separate magnetic particles of less than 0.03 microns in diameter.

The ferromagnetic carrier particles must generally be coated in order to provide a reactive substrate for attaching chemical functional groups to bind enzymes or antibodies, for example. Silane polymers are often used for this purpose. Particles described in U.S. Patent No. 3,933,997 are coated with silanes capable of reacting with anti-digoxin antibodies to chemically couple the antibodies to the carrier particles. Various silane couplings are discussed in U.S. Patent No. 3,652,761, which is hereby incorporated by reference. Procedures for silanization known in the art generally differ from each other in the media chosen for the polymerization of silane and its disposition on reactive surfaces. The medium is generally an organic solvent such as toluene (H.W., Weetall, In: Methods of Enzymology, K. Mosbach (ed), 44:134-148, 140 (1976)), methanol (U.S. Patent No. 3,933,997) or chloroform (U.S. Patent No. 3,652,761).

Silane depositions from aqueous alcohol and aqueous solutions with acid have also been used. H.W. Weetall, In: Methods in Enzymology, supra, p.139 (1976).

There are several drawbacks to silane-coated particles. For example, the dehydration methods used to dry the coatings, such as air and/or oven drying, allow the silanized surfaces of the carrier particles to contact each other, potentially resulting in interparticle bonding, including cross-linking between particles by siloxane formation, van der Waals interactions or physical adhesion between adjacent particles. This interparticle bonding yields covalently or physically bonded aggregates of silanized carrier particles of considerably larger diameter than individual carrier particles. Such aggregates have low surface area per unit weight and hence, a low capacity for coupling with molecules such as antibodies, antigens or enzymes. Such aggregates also have gravitational settling times which are too short for many applications.

Magnetic particles capable of binding bioaffinity reagents are useful in separating desired biological components from a sample, for example, in radioimmunoassay. Radioimmunoassay (RIA) is a term used to describe methods for analyzing the concentrations of substances involving a radioactively labeled substance which binds to an antibody. The amount of radioactivity bound is altered by the presence of an unlabeled test substance capable of binding to the same antibody. The unlabeled substance, if present, competes for binding sites with the labeled substance and thus decreases the amount of radioactivity bound to the antibody. The

decrease in bound radioactivity can be correlated to the concentration of the unlabeled test substance by means of a standard curve. An essential step of RIA is the separation of bound and free label which must be accomplished in order to quantitative the bound fraction.

A variety of conventional separation approaches have been applied to RIA including coated tubes, particulate systems, and double antibody separation methods. Coated tubes, such as described in U.S. Patent No, 3.646,346 allow separation of bound and free label without centrifugation but suffer from two major disadvantages. First, the surface of the tube limits the amount of antibody that can be employed in the reaction. Second, the antibody is far removed (as much as 0.5 cm) from the antigen, slowing the reaction between the antibody and antigen. G.M. Parsons, In: Methods in Enzymology, J.F. Langone (ed), 73:225 (1981); P.N. Nayak, The Ligand Quarterly, 4(4):34 (1981).

Antibodies have been attached to particulate systems to facilitate separations. U.S. Patent Nos. 3,652,761 and 3,555,143. Such systems have large surface areas permitting nearly unlimited amounts of antibody to be used, but the particulates frequently settle during the assay. The tube frequently must be agitated to achieve even partial homogeneity P.M. Jacobs, The Ligand Quarterly, 4(4):23-33 (1981). Centrifugation is still required to effect complete separation of bound and free label.

Antibodies may react with labeled and unlabeled molecules followed by separation using a second antibody raised to the first antibody. The technique, termed the

doubled antibody method, achieves homogeneity of antibody during reaction with label but requires an incubation period for reaction of first and second antibodies followed by a centrifugation to pellet the antibodies.

Antibodies have been attached to magnetic supports in an effort to eliminate the centrifugation steps in radioimmunoassays for nortriptyline, methotrexate, digoxin, thyroxine and human placental lactogen R.S. Kamel et al., Clin. Chem., 25(12):1997-2002 (1979); R.S. Kamel and J. Gardner, Clin. Chem. Acts, 89:363-370 (1978); U.S. Patent No., 3,933,997; C. Dawes and J. Gardener, Clin. Chem. Acts, 86:353-356 (1978); D.S. Ithakissios et al., Clin, Chem. Acts 84:69-84 (1978); D.S. Ithakissios and D.O. Kubiatowicz, Clin. Chem. 23(11):2072-2079 (1977); and L. Nye et al., Clin. Chem. Acts., 69:387-396 (1976), the teachings of the above are hereby incorporated by reference. Such methods suffer from large particle sizes (10-100 microns in diameter) and require agitation to keep the antibody dispersed during the assay. Since substantial separation occurs from spontaneous settling in the absence of a magnetic field these previous methods are in fact only magnetically assisted gravimetric separations. Davies and Janata in U.S. Patent No. 4,177,253 employed magnetic materials such as hollow glass or polypropylene (4-10 microns in diameter) with magnetic coatings (2 microns 10 microns thick) covering a proportion of the particle surface. Antiestradiol antibodies were coupled to such particles and their potential usefulness in estradiol RIAs was demonstrated. While this approach may have over come the problem of settling, the particle size and the

magnetic coating nonetheless present limitations on surface area and hence limitations on the availability of sites for antibody coupling.

Magnetic separations have been applied in other biological systems besides RIA. Several nonisotopic immunoassays, such as fluoroimmunoassays (FIA) and enzyme-immunoassays (EIA) have been developed which employ antibody-coupled (or antigen coupled) magnetic particles. The principle of competitive binding is the same in FIA and EIA as in RIA except that fluorophores and enzymes, respectively, are submitted for radioisotopes as label. By way of illustration, M. Pourfarzaneh et al., and R.S. Kamel et al., developed magnetizable solid-phase FIA's for cortisol and phenytoin, respectively, utilizing ferromagnetic cellulose/iron oxide particles to which antibodies were coupled by cyanogen bromide activation. M. Pourfarzaneh et al., Clin. Chem., 26(6):730-733 (1980); R.S. Kamel et al., Clin. Chem., 26(9):1281-1284 (1980).

A non-competitive solid phase sandwich technique EIA for the measurement of IgE was described by J.L. Guesdon et al., in J. Allergy Clin. Immunol., 61(1):23-27 (1978). By this method, anti-IgE antibodies coupled by glutaraldehyde activation to magnetic polyacrylamide agarose beads are incubated with a test sample containing IgE to allow binding. Bound IgE is quantitated by adding a second anti-IgE antibody labeled with either alkaline phosphatase or B-galactosidase. The enzyme labeled second antibody complexes with IgE bound to the first antibody, forming the sandwich, and the particles are separated magnetically. Enzyme activity associated with

the particles, which is proportional to bound IgE is then measured permitting IgE quantitation.

A magnetizable solid phase non-immune radioassay for vitamin B12 has been reported by D.S. Itha issios and D.O. Kubiatowicz Clin. Chem., 23(11):2072-2079 (1977). The principle of competitive binding in non-immune radioassays is the same as in RIA with both assays employing radioisotopic labels. However, while RIA is based on the binding or interaction of certain biomolecules like vitamin B12 with specific or non-specific binding, carrier, or receptor proteins. The magnetic particles of Ithakissios and Kubiatowicz were composed of barium ferrite particles embedded in a water-insoluble protein matrix.

In addition to their use in the solid phase biological assays just described, magnetic particles have been used for a variety of other biological purposes. For example, magnetic particles have been used in cell sorting systems to isolate select viruses, bacteria and other cells from mixed populations. U.S. Patent Nos., 3,970,518; 4,230,685; and 4,267,234, the teachings of which are hereby incorporated by reference. They have been used in affinity chromatography systems to selectively isolate and purify molecules from solution and are particularly advantageous for purification from colloidal suspensions. K. Mosbach and L. Anderson, Nature 170:259-261 (1977), hereby incorporated by reference. Magnetic particles have also been used as the solid phase support immobilized enzyme systems. Enzymes coupled to magnetic particles are contacted with substrates for a time sufficient to catalyze the biochemical reaction.

Thereafter, the enzyme can be magnetically separated from products and unreacted substrate and potentially can be reused. Magnetic particles have been used as supports for a-chymotrypsin, β-galactosidase U.S. Patent No. 4,152,210), hereby incorporated by reference) and glucose isomerase (U.S. Patent No. 4,343,901, hereby incorporated by reference) in immobilized enzyme systems.

Summary of the Invention

The present invention relates to magnetically responsive particles coated with a carbohydrate or vinyl polymer capable of binding bioaffinity adsorbents, and to their use in the separation of biological molecules from, or directed movement of the molecules in, the surrounding medium. Methods and compositions for preparing and using the coated magnetic particles are provided.

The magnetic particles comprise a magnetically responsive metal, metal alloy, or metal oxide core surrounded by a polymer coating which is adsorbed or covalently bound to the particle. A wide variety of bioaffinity adsorbents can be covalently bonded to the polymer coating through selected coupling chemistries.

More particularly, the invention relates to methods for the preparation of magnetically responsive particles comprising a metal, metal alloy or metal oxide core and a carbohydrate or vinyl polymer coating having an aliphatic moiety and an organic functionality to which a variety of organic and/or biological molecules can be coupled. The particles, coupled or uncoupled, can be dispersed in aqueous media forming a colloidal dispersion which is stable, that is, the particles resist rapid gravitational

settling. The particles can be reclaimed from the media by applying a magnetic field.

Preferably, the particles are superparamagnetic; that is, they exhibit no reminent magnetization after removal of a magnetic aggregate which allows the particles to be redispersed without magnetic aggregate formation.

The magnetically responsive particles of the invention may be coupled to biological or organic molecules with affinity for, or the ability to adsorb, or which interact with, certain other biological or organic molecules. Particles so coupled may be used in a variety of in vitro or in vivo systems involving separations steps or the directed movement of coupled molecules to particular sites, including immunological assays, other biological assays, biochemical or enzymatic reactions, affinity chromatographic purification, cell sorting and diagnostic and therapeutic uses.

The present magnetic particles provide superior composition, size, surface area, coupling versatility, settling properties and magnetic behavior for use in biological separations. The magnetic particles of this invention are suitable for many of the assays, enzyme immobilization, cell sorting and affinity chromatography procedures reported in the literature and, in fact, overcome many of the problems associated with particle settling and reuse experienced in the past with such procedures.

Detailed Description of the Invention

The magnetically responsive particles of this invention overcome problems associated with the size, surface area, gravitational settling rate and magnetic character of previously developed magnetic particles. Gravitational settling times in excess of about 1.5 hours can be achieved with the present magnetic particles. The gravitational settling time is defined to be the time for the turbidity of a dispersion of particles to fall by fifty percent in the absence of a magnetic field.

The present magnetic particles comprise a core of a magnetically responsive metal, metal alloy or metal oxide, coated with a carbohydrate or vinyl polymer which is capable of binding bioaffinity agents. The term "magnetically responsive particle" or "magnetic particle" is defined as any particle dispersible or suspendible in aqueous media without significant gravitational settling, and separable from suspension by application of a magnetic field. The term "magnetocluster" is a synonym of "magnetically responsive particle" and "magnetic particle".

The term "magnetic core" is defined as a crystal or group (or cluster) of crystals of a transition metal, alloy or metal oxide having ferrospinel structure and comprising trivalent and divalent cations of the same or different transition metals or magnetic metal crystals group. Metals, alloys and oxides which are useful as magnetic core material in the present invention include the metals, alloys and oxides based on metals which appear in the Periodic Table in Groups 4a and b, 5a and b, 6a and 7a. These include, for example, divalent

transition metals, such as iron, magnesium, manganese, cobalt, nickel, zinc and copper, alloys of these metals such as iron alloys or oxides (e.g., iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide), cobalt ferrite, samarium cobalt, barium ferrite, and aluminum-nickel-cobalt and metal oxides including magnetite (Fe_3O_4), hematite (Fe_2O_3) and chromium dioxide. By way of illustration, a magnetic core may be comprised of a cluster of superparamagnetic crystals of iron oxide, or a cluster of ferromagnetic crystals of irons or oxide, or may consist of a single ferromagnetic crystal of an iron oxide.

The present particles are preferably between about 0.005 and about 1.5 microns in diameter, and have a surface area of from about 100 to 150 m/gm, which provides a high capacity for bioaffinity adsorbent coupling. Magnetic particles of this size range overcome the rapid settling problems of larger particles, but obviate the need for large magnets to generate the magnetic fields and magnetic field gradients required to separate smaller particles. For example, magnets used to effect separations of the magnetic particles of this invention need only generate magnetic fields between about 100 and about 1000 Oersteds. Such fields can be obtained with permanent magnets which are smaller than the container which holds the dispersion of magnetic particles and, thus, are suitable for benchtop use.

Particles with superparamagnetic behavior are preferred since superparamagnetic particles do not exhibit the magnetic aggregation associated with

ferromagnetic particles and permit redispersion and reuse. The term "superparamagnetism" is defined as that magnetic behavior exhibited by iron, metal alloys or metal oxides having a crystal size of less than about 300A, which behavior is characterized by responsiveness to a magnetic field without resultant permanent magnetization.

Ferromagnetic particles may be useful in certain applications of the invention. The term "ferromagnetism" is defined as that magnetic behavior exhibited by iron oxides with a crystal size greater than about 500A, which behavior is characterized by responsiveness to a magnetic field with resultant permanent magnetization.

The particles or crystals are then coated with a polymeric material capable of adsorptive or covalently bonding to the magnetic particles. Polymer useful for the present coated particles are carbohydrate polymers and vinyl polymers which can be coated or adsorbed onto the magnetic particle and, crosslinked in situ on the particle surface, and must be able to be functionalized in a manner that allows the polymer to form covalent bonds with bioaffinity or chemical affinity adsorbents. The polymer is post-functionalized or derivitized with an aliphatic "spacer arm" which is terminated with an organic functional group capable of coupling with bioaffinity adsorbents. The "spacer arm" is an aliphatic hydrocarbon having from about 3 to about 20 carbon atoms. The purpose of the spacer arm is to provide a non-reactive linker (or spacer) between the organic group and the polymer chain. The organic group is a reactive group such as amines (NH_2), carboxyl groups (COOH),

cyanate (CN), phosphate (PO_3H), sulfate (SO_3H), thiol (SH) or hydroxyl groups (OH).

Particularly useful carbohydrate polymers include polysaccharides such as carboxymethyl cellulose, dextran, agarose and cellulose. Particularly useful vinyl polymers include polyvinyl alcohol, polyvinyl pyrrolidone, polyethylene oxide, polyacrylonitrile and polyacrylamide. In one embodiment of the present invention, carboxymethyl cellulose is coated onto the magnetic particle of choice, and, crosslinked. Compounds which are useful as crosslinkers include, for example, 2, 3-dibromopropanol, cyanogen bromide, isocyanates, carbodiimide and glutaraldehyde.

The coated particle functionalized in a manner that allows the polymer to form covalent bonds with bioaffinity or chemical affinity adsorbents. In this method, the polysaccharide is first activated by reaction with an agent such as cyanogen bromide. The organic functionality is then linked to the activated polymer, for example, by adding an amino compound such as 1, 6 hexanediamine; a carboxyl compound such as lysine, or a hydroxy compound such as 6-amino hexanol.

The present magnetic particles are prepared according to the following general procedure: metal salts are precipitated in a base to form fine magnetic metal oxide crystals. The crystals are redispersed, then washed in water and in an electrolyte. Magnetic separations can be used to collect the crystals between washes if the crystals are superparamagnetic.

In one embodiment of the present invention, super-paramagnetic iron oxide particles are made by

precipitation of divalent (Fe^{2+}) and trivalent (Fe^{3+}) iron salts, for example, $FeCl_2$ and $FeCl_3$, in base. The ratio of Fe^{2+} and Fe^{3+} can be varied without substantial changes in the final product by increasing the amount of Fe^{2+} while maintaining a constant molar amount of iron. A Fe^{2+}/Fe^{3+} ratio of about 2:1 to about 4:1 is useful in the present invention; a ratio of about 2:1 $Fe^{2+}:Fe^{3+}$ is particularly useful. An Fe^{2+}/Fe^{3+} ratio of 1:1 produces magnetic particles of slightly inferior quality to those resulting from the higher Fe^{2+}/Fe^{3+} ratios, the particle size is more heterogeneous than that resulting from Fe^{3+}/Fe^{2+} of 2:1 or 4:1.

In this embodiment, aqueous solutions of the iron salts are mixed in a base, such as ammonium hydroxide which results in the formation of a crystalline precipitate of superparamagnetic iron oxide. The precipitate is washed repeatly with water by magnetically separating it and redispersing it until a neutral pH is reached. The precipitate is then washed once in an electrolytic solution, e.g., a sodium chloride solution. The electrolyte wash step is important to insure fineness of the iron oxide crystals.

The repeated use of magnetic fields to separate the iron oxide from suspension during the washing steps is facilitated by the superparamagnetic properties of the crystals. Regardless of how many times the particles are subjected to magnetic fields, they never become permanently magnetized and consequently can be redispersed by mild agitation. Permanently magnetized (ferromagnetic) metal oxides cannot be prepared by this washing procedure as they tend to magnetically aggregate

after exposure to magnetic fields and cannot be homogeneously redispersed.

In another embodiment of the present invention, the present particles can be made by precipitating metal powders and reducing the particle size by ball milling the resulting precipitate. In this process, the metal powder is precipitated from an aqueous solution of, for example, Fe^{+2} or Fe^{+3} salt with sodium borohydride. The resulting properties of the metal powder are unaffected by the valance of the counter ion or iron metal salt selected. Complete precipitation occurs spontaneously upon borohydride addition. The magnetic metal powder is then collected by filtration and washed with water to remove all soluble salts. The particle is added as an aqueous slurry in a concentration of about 1-25% to a commercial ball mill filled half way with 1/4" stainless steel balls and milled for 3-30 days. At the completion of the milling period, a superparamagnetic metal slurry is formed and treated as the magnetite described in the previous section.

Other divalent transition metal salts such as magnesium, manganese, cobalt, nickel, zinc and copper salts may be substituted for iron salts in the precipitation or milling procedure to yield magnetic metal oxides. For example, the substitution of divalent cobalt chloride ($COCl_2$) for $FeCl_2$ in the above procedure produced ferromagnetic metal oxide particles. Ferromagnetic metal oxide particles such as those produced with $COCl_2$ can be washed in the absence of magnetic fields by employing conventional techniques of centrifugation or filtration between washings to avoid magnetizing the particles. As

long as the resulting ferromagnetic metal oxides are of sufficiently small diameter to remain dispersed in aqueous media, they can also be coated with a polymer and coupled to bioaffinity adsorbents for use in systems requiring a single magnetic separation, e.g., certain radioimmunoassays. Ferromagnetism limits particle usefulness in those applications requiring redispersion or reuse.

In one embodiment of the present invention, the coating around the magnetic particle core is a polymer of carboxymethyl cellulose. The polymerization is performed by redispersing the magnetic particle in a neutral aqueous solution, adding carboxymethyl cellulose, then crosslinking, e.g., with 2,3 dibromopropanol. The polymer can be activated with cyanogen bromide, for example, and functional groups can then be added, e.g., by adding 1, 6 hexanediamine, thereby forming a coupled magnetically responsive particle. The term "coupled magnetically responsive particle" or "coupled magnetic particle" is defined as any magnetic particle to which one or more types of bioaffinity adsorbents are coupled by covalent bond, which covalent bonds may be amide, ester, ether sulfonamide, disulfide, azo or other suitable organic linkages depending on the functionalities available for bonding on both the coat of the magnetic particle and the bioaffinity adsorbents.

Preferred magnetically responsive particles of the present invention have metal oxide cores composed of clusters of superparamagnetic crystals affording efficient separation of the particles in low magnetic fields (100-1000 Oersteads) while maintaining

superparamagnetic properties. Aggregation of particles is controlled during particle synthesis to produce particles which are preferably small enough to avoid substantial gravitational settling over times sufficient to permit dispersions of the particles to be used in an intended biological assay or other application. The advantage of having superparamagnetic cores in magnetically responsive particles is that such particles can be repeatedly exposed to magnetic fields. Superparamagnetic particles do not exhibit reminent magnetization and have no coercive strength, and, therefore, do not magnetically aggregate, thus, the particles can be redispersed and reused. Even after coating, preferred particles of the invention having cores made up of clusters of crystals exhibit a remarkably high surface area per unit weight and a generally corresponding high coupling capacity, which indicates that such particles have an open or porous structure.

The magnetic particles of this invention can be covalently bonded by conventional coupling chemistries to bioaffinity adsorbents. Several coupling reactions can be performed. For example:

(a) If the ligand contains an amino group, it can be coupled directly to the activated polysaccharide. A different functionality can be introduced, for example, a spacer arm can be introduced by sequential reaction with a diaminoalkane and glutaraldehyde. The amino group on the ligand can then be coupled to the free aldehyde group.

(b) If the ligand contains an aldehyde group instead of an amino group, it can be coupled directly to the free amino group of the diaminoalkane.

(c) Ligands may be coupled to polyacrylamide by displacing the amide group of the polyacrylamide by heating with a diaminoalkane, followed by reaction with glutaraldehyde. The Schiff base that results from the reaction of glutaraldehyde with an amino group may be stabilized by reduction with sodium cyanoborohydride without affecting the aldehyde group. The ligand can then be coupled to the aldehyde group.

(d) Another method of activating polyacrylamide is to form the hydrazide derivative by reaction with hydrizine hydrate. When an amino, aldehyde, or hydrazide group is incorporated onto the solid support, the support becomes activated so that ligands may be attached through amino, carboxyl, phenolic, or imidazole groups.

The term "bioaffinity adsorbent" is defined as any biological or other organic molecule capable of specific or nonspecific binding or interaction with another biological molecule, which binding or interaction may be referred to as "ligand/ligate" binding or interaction and is exemplified by, but not limited to, antibody/antigen, antibody/hapten, enzyme/substrate, carrier protein/substrate, lectin/carbohydrate, receptor/hormone, receptor/effector or repressor/inducer bindings or interactions.

The coupled magnetic particles of the present invention can be used in immunoassays or other binding assays for the measurement of analytes in solution. The term "immunoassay" is defined as any method for measuring the concentration or amount of an analyte in a solution

based on the immunological binding or interaction of a polyclonal or monoclonal antibody and an antigen, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally directly proportional to the amount of analyte originally in the solution. Label may be in the antigen, the antibody, or in double antibody methods, the second antibody. Immunoassays are exemplified by, but are not limited to, radioimmunoassays (RIA), immunoradiometric assays (IRMA), fluoroimmunoassays (FIA), enzyme immunoassays (EIA), and sandwich method immunoassays. The bioaffinity adsorbent can include, for example, antibodies, antigens, haptens, enzymes, apoenzymes, enzymatic substrates, enzymatic inhibitors, cofactors, binding proteins, carrier proteins, compounds bound by binding proteins, compounds bound by carrier proteins, lectins, monosaccharides, polysaccharides, hormones, receptors, repressors and inducers.

Such assays are preferably carried out by mixing a sample containing an unknown concentration of analyte with a known amount of labeled analyte in the presence of magnetic particles coupled to a bioaffinity adsorbent capable of binding to, or interacting with, both unlabeled and labeled analyte, allowing the binding or interaction to occur, magnetically separating the

particles, measuring the amount of label associated with the magnetic particles and comparing the amount of label to a standard curve to determine the concentration of analyte in the sample.

The term "binding assay" or "non immune assay" is defined as any method for measuring the concentration or amount of an analyte in solution based on the specific or nonspecific binding or interaction, other than antibody/antigen binding or interaction, or a bioaffinity adsorbent and another biological or organic molecule, which method (a) requires a separation of bound from unbound analyte; (b) employs a radioisotopic, fluorometric, enzymatic, chemiluminescent or other label as as the means for measuring the bound and/or unbound analyte; and (c) may be described as "competitive" if the amount of bound measurable label is generally inversely proportional to the amount of analyte originally in solution or "non-competitive" if the amount of bound measurable label is generally originally in solution.

The magnetic particles of this invention are useful in immobilized enzyme systems, particularly where enzyme recycling is desired. The term "immobilized enzyme system" is defined as any enzymatically catalyzed biochemical conversion or synthesis or degradation wherein the enzyme molecule or active site thereof is not freely soluble but is adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said method. In this embodiment, enzymatic reactions are carried out by dispersing enzyme-coupled magnetic particles in a reaction mixture

containing one or more substrates, under conditions sufficient for the reaction between the enzyme and substrate to occur, magnetically separating the enzyme-magnetic particle from the reaction mixture containing products and unreacted substrates and, if desired, redispersing the particles in fresh substrates thereby reusing the enzyme.

Affinity chromatography separations and cell sorting can be performed using the magnetic particles of this invention. The term "affinity chromatography" is defined as a method for separating, isolating, and/or purifying a selected molecule from its surrounding medium on the basis of its binding or interaction with a bioaffinity adsorbent adsorptively or covalently bound to a solid phase support, which support is suspended in or contacted with the surrounding medium and which may be reclaimed or separated from said medium by dispersing bioaffinity adsorbent coupled magnetic particles in solutions or suspensions containing molecules or cells to be isolated and/or purified, allowing the bioaffinity adsorbent and the desired molecules or cells to interact, magnetically separating the particles from the solutions or suspension and recovering the isolated molecules or cells from the magnetic particles.

It is further contemplated that the magnetic particles of this invention can be used in <u>in vivo</u> systems for the diagnostic localization of cells or tissues recognized by the particular bioaffinity adsorbent coupled to the particle and also for magnetically directed delivery of therapeutic agents coupled to the particles to pathological sites.

Magnetic separation times of less than about ten minutes can be achieved with magnetic particles of the invention by contacting a vessel containing a dispersion of the particles with a pole face of a permanent magnet no larger in volume than the volume of the vessel. Magnetic separation time is defined to be the time for the turbidity of the dispersion to fall by 95 percent.

Furthermore, the use of functionalized carbohydrate polymers as the coating surrounding the metal oxide core of the magnetic particles described herein make possible the coupling of a wide variety of molecules under an equally wide variety of coupling conditions compared to other magnetic particle coatings known in the art with more limited coupling functionalities. Still further, the use of hydrogel polymers in conjunction with magnetic metal powders make the particles more stable in a gravity field, and separate faster in a magnetic field than other technologies involving magnetic separation.

The invention is further illustrated by the following Examples.

EXAMPLES

Example 1: Preparation of Superparamagnetic Magnetite Particles

200 grams (1.58 moles) of ferrous chloride (VWR Scientific) and 325 grams (2.0 moles) of ferric chloride were dissolved in 3 liters of water. 2000 grams of ammonium hydroxide (VWR Scientific) concentrate were added at a rate of 50 ml/minute under constant agitation, during which time the temperature of the solution was

kept between 25 and 40°C. After the addition of the ammonium hydroxide was complete, the magnetic particle (Fc_3O_4) aqueous slurry was allowed to cool to room temperature.

Example 2: Preparation of Amine-Functional Carboxymethyl Cellulose-Coated Magnetic Particles

4 moles of $FeCl_3$, 2 moles of $FeCl_2$, were dissolved in 4 L of distilled water and precipitated with 16 moles of NH_4OH according to the procedure set out in Example 1. The precipitate was washed five times with water. Carboxymethyl cellulose (CMC) was added to the precipitate in the following ratio: dry Fe_3O_4: CMC = 2:1 weight percent. The mixture was loaded into a ball mill consisting of a 16 oz. glass jar which was loaded 1/2 full with 4 mm stainless steel beads, and milled overnight. 2,3-dibromopropanol was added in the following ratio: CMC: dibromopropanol = 5:1 weight percent, and the mixture was milled for two hours in the ball mill. Cyanogenbromide (CNBr) was added to activate the CMC in the following ratio: CMC: CNBr = 1:0.1 weight percent, and the mixture was milled for two hours in the ball mill. 1,6 hexanediamine was added in the following ratio: CMC: 1,6 hexanediamine = 5:1 weight percent, and the mixture was milled in the ball mill for 1/2 hour. The resulting mill base was then washed five times with water, and the coated particles collected by addition of an external magnetic field of 2000 gauss and decanting the aqueous waste.

The resulting material was a CMC coated, amine functional magnetic microsphere that can be suspended in aqueous media for a period of greater than 24 hours in the absence of a magnetic field, and can be separated in the presence of a 100 grams, or greater, magnetic field in under 10 seconds.

Example 3: Preparation of Carboxy-Functional CMC-Coated Magnetic Particles

The procedure described in Example 2 was followed except that lysine was used in place of 1,6 hexanediamine to produce carboxyl-functional magnetic microspheres.

Example 4: Preparation of Hydroxyl-Functional CMC-Coated Magnetic Particles

The procedure described in Example 2 was followed except that 6-amino hexanol was used in place of 1, 6 hexanediamine to produce hydroxyl-functional magnetic microspheres.

Example 5: Preparation of Crosslinked CMC-Coated Magnetic Particles

The procedure described in Example 2 was followed except that epichlorohydrin was used in place of 2.3-dibromoprepanol to crosslink the carboxymethyl cellulose to the magnetic microsphere.

In this process, magnetite (Fe_3O_4) was precipitated as described (Example 1) and washed 5X with water. Carboxymethyl cellulose was added as described in Example 2, then epichlorohydrin was added in the following ratio: CMC: epichlorohydrin = 4:1 wt%, and the mixture was

milled in the ball mill for 2 hours. Cyanogen bromide was added (CMC: CNBr = 1:0.1% by weight) and the mixture milled in a ball mill for 2 hours. 1,6 hexanediamine was then added, and the product was milled, washed and collected as described in Example 2.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, by no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following Claims.

CLAIMS

1. A coated magnetically responsive particle comprising:
   a) a core particle comprising a magnetically-responsive metal, metal alloy or metal oxide; and
   b) a polymer coating said particle, wherein the polymer coating is a carbohydrate or vinyl polymer capable of binding at least one type of bioaffinity adsorbent.

2. A coated magnetically responsive particle of Claim 1, wherein the core comprises a metal, metal alloy or metal oxide selected from the group consisting of iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide.

3. A coated magnetically responsive particle of Claim 2 wherein the core is magnetite.

4. A coated magnetically responsive particle of Claim 1, having a particle size of from about 0.005 to about 1.5 microns in diameter.

5. A coated magnetically responsive particle of Claim 1, wherein the polymer is selected from the group consisting of carboxymethyl cellulose, dextran, agarose, cellulose, polyvinyl alcohol, polyvinyl

pyrrolidone, polyethylene oxide, polyacrylonitrile and polyacrylamide.

6. A coated magnetically responsive particle of Claim 5 wherein the polymer is the carboxymethyl cellulose.

7. A coated magnetically responsive particle of Claim 1 which is superparamagnetic.

8. A coated magnetically responsive particle comprising:

a) a core particle comprising a magnetically responsive metal, metal alloy or metal oxide;

b) a carbohydrate or vinyl polymer coating the particle, said polymer having organic functional groups attached thereto; and

c) a bioaffinity adsorbent covalently coupled to the polymer coating.

9. A coated magnetically responsive particle of Claim 8, wherein the core particle is metal, metal alloy or metal oxide selected from the group consisting of: iron, magnetite, iron magnesium oxide, iron manganese oxide, iron cobalt oxide, iron nickel oxide, iron zinc oxide and iron copper oxide.

10. A coated magnetically responsive particle of Claim 9 wherein the core comprises magnetite.

11. A coated magnetically responsive particle of Claim 8 wherein the polymer coating comprises carboxymethyl cellulose.

12. A coated magnetically responsive particle of Claim 8 wherein the bioaffinity adsorbent is selected from the group consisting of: antibodies, antigens, enzymes and specific binding proteins.

13. A coated magnetically responsive particle of Claim 10 which is superparamagnetic.

14. A coated magnetically responsive particle of Claim 8, wherein the organic functional groups are selected from the group consisting of: amino, carboxyl, hydroxyl, sulfate, phosphate, cyanate and thiol groups.

15. A coated magnetically responsive particle of Claim 8 having a mean diameter of from about 0.005 to about 1.5 microns.

16. A magnetically responsive particle comprising a superparamagnetic metal oxide core surrounded by a coat of carboxymethyl cellulose polymer to which bioaffinity adsorbents can be covalently coupled, the metal oxide core comprising a group of crystals of metal oxide, and the particle having a mean diameter of about 0.005 to about 1.5 microns.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/03409

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: B 03 C 1/00, G 01 N 33/543, H 01 F 1/37, H 01 F 1/28

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	B 03 C, H 01 F

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	US, A, 4452773 (R.S. MOLDAY) 5 June 1984 see claims 1-4,6,8,11 --	1-5,7-10,12-15
X	DE, A, 3709852 (SILICA GEL GMBH) 6 October 1988 see claims 1,2,6,9,19; column 6, line 67 - column 7, line 9 --	1-5,7-10,12-15
X	FR, A, 2334106 (INSTITUT PASTEUR) 1 July 1977 see claims 1-4 --	1-3,5,8-10, 12,14
X	WO, A, 83/03426 (GAMBRO LUNDIA AB) 13 October 1983 see claims 1-4; page 3, lines 9-16; page 6, lines 16-18 ------	1-5,7-10,12, 13,15

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13th September 1990	03. 10. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	H. Ballesteros

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9003409 SA 38117

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/09/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A- 4452773	05-06-84	CA-A- 1217934	17-02-87
DE-A- 3709852	06-10-88	None	
FR-A- 2334106	01-07-77	DE-A,C 2654723	23-06-77
		GB-A- 1577956	29-10-80
		JP-A- 52082723	11-07-77
		SE-A- 7613487	03-06-77
		SE-B- 441393	30-09-85
		SE-A- 8000427	17-01-80
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		SE-A- 8201972	30-09-83
		US-A- 4687748	18-08-87